American Journal of Medical Genetics 68:445–449 (1997)

Transmission Electron Microscopy of Chromosomes by Longitudinal Section Preparation: Application to Fragile X Chromosome Analysis

Guang Y. Wen,* Edmund C. Jenkins, Xiu-Lan Yao, David Yoon, W. Ted Brown, and Henryk M. Wisniewski

New York State Institute for Basic Research in Developmental Disabilities, Staten Island

We developed a method for the preparation of ultrathin longitudinal sections of chromosomes enabling TEM studies of whole chromosomes. By using a novel "repeat chill" method of exposing the glass slide and plastic block interface to liquid nitrogen, it was possible to separate consistently hardened epoxy resin-embedded chromosome spreads from glass slides for ultrathin longitudinal sectioning of entire spreads and of individual chromosomes. The method was applied to analyze the fragile X chromosome. The ultrastructure of centromeres, telomeres, fragile sites and other chromosomal areas can now be studied in detail. Am. J. Med. Genet. 68:445–449, 1997.

KEY WORDS: transmission electron microscopy (TEM); ultrathin longitudinal sections; chromosomes; fragile X chromosome; FISH; liquid nitrogen

INTRODUCTION

Previously, TEM chromosome studies were limited to whole mounts and random partial longitudinal sections [Rattner, 1987; Squarzoni et al., 1994; Wandall, 1994]. In order to overcome these limitations, we have developed a method that allows for consistently high-quality preparations of ultrathin longitudinal sections for transmission electron microscopy (TEM). This allows for analysis of the whole of any or all chromosomes within a spread.

MATERIALS AND METHODS

Cytogenetics

Fragile X chromosomes from EBV-transformed lymphoblastoid cultures from a known full-mutation fragile X male individual were prepared according to previously described protocols [Krawczun et al., 1986, 1990; Jenkins et al., 1992]. The fragile X cytogenetic frequency was 20%. X chromosomes on glass slides were hybridized with a biotinylated α-satellite probe, specific for the X chromosome centromere, using a FISH protocol that was provided with the probe and its appropriate kit from ONCOR (Gaithersburg, MD). X chromosome identification and documentation of digitized color images, for future relocation within the TEM longitudinal ultrathin sections, was carried out as described previously [Yao et al., 1995].

Transmission Electron Microscopy

The X and Y coordinates were initially indicated by marking the edges of the microscope slide. The intercept of X and Y coordinates corresponding to the location of the mitotic figure containing the preidentified X chromosome was marked with a "+" on the underside of the glass slide with a diamond pen. The + sign and the mitotic figure can be focused alternatively to ensure accuracy in placing the + sign as well as 100% relocation efficiency. Osmium tetroxide and tannic acid impregnation were employed according to a previously described protocol by Sanchez-Sweatman [1993]. It was found to be critically important to filter all fixatives, buffers, tannic acid solution, and distilled water with a 0.22-μm Millipore filter to provide clean preparations by removing precipitates from all the above solutions. Chromosomes were postfixed, following original fixation with 3:1 methanol:acetic acid, by covering the preparation with a few drops of 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB) pH 7.4, containing 0.1 M sucrose for 30 minutes/overnight, rinsed 3 times with PB for a total of 4′ (minutes). They were further postfixed with 1% osmium tetroxide in 0.1 M PB for 10′,

Contract grant sponsor: New York State Office of Mental Retardation and Developmental Disabilities; Contract grant sponsor: The Maternal and Child Health Program; Contract grant number: MCJ360587; Contract grant sponsor: Health Resources and Services Administration, Department of Health and Human Services.

*Correspondence to: Dr. G.Y. Wen, New York State Institute for Basic Research in Developmental Disabilities, 1050 Forest Hill Road, Staten Island, NY 10314.

Received 6 February 1996; Accepted 3 May 1996

rinsed 3 times with distilled water for 4′, covered with freshly prepared 2% tannic acid in distilled water for 10′, and rinsed 3 times in distilled water for 4′. This osmium-tannic acid postfixation procedure was repeated 3 times. The chromosomes were then dehydrated for 10–15 minutes each through 50, 70, 85, 95, and twice in 100% ethanol in a Coplin jar.

Slides with preidentified and prelocated X chromosomes, including fragile X chromosomes, were then placed in propylene oxide (PO) twice for 10′ each, followed by infiltration in a 1:1 mixture of PO and Spurr medium (SM) [Spurr, 1969] for 60′. They were then placed in pure SM and held overnight at 4°C in a tightly closed Coplin jar to prevent effects of humidity on the SM. The sample was then embedded/covered with an upside-down Beem capsule (with its tip pointing upward) containing SM. The center of the Beem capsule containing SM was positioned to face/match the "+" mark made on the underside of the slide with the diamond pen to facilitate relocation of the preidentified X chromosome in the plastic block after polymerization.

The Beem capsule covering the X chromosome was placed in a 70°C oven for polymerization over 12–24 hours. Before separating the chromosomes from the slide, a knife-scratch mark was made at 9, 12, and 3 o'clock positions, on the plastic tissue block for correct orientation indicating that the 12:00 position corresponded to the upper side of the digitized image of the chromosomes so that the pre-identified X chromosome could be relocated for trimming of the block and ultrathin sectioning.

An adequate amount of liquid nitrogen was poured into a plastic petri dish so that it would cover a glass slide. The plastic SM tissue block attached to the glass slide was then immersed for 6–7″ (seconds) in liquid nitrogen to chill the slide/tissue block interface before separation was attempted. As some pieces of glass slide usually remained attached to the surface of the plastic tissue block, it was warmed up for 2–3′ at room temperature, or with finger-touching or in a 70°C oven and repeat-chilled by reimmersion in liquid nitrogen for 6–7″. This procedure was repeated until all of the glass pieces on the surface of the plastic tissue block were removed with the tip of a razor blade or with fine forceps. All remaining pieces of glass slide covering the chromosome spread containing the preidentified X, were removed to prevent diamond knife damage during ultramicrotoming. We refer to this procedure as the "repeat chill" method.

Ultrathin EM sections at thicknesses of 600–900 Å (interference color of silver) were cut and placed on formvar-coated 75 mesh copper grids. The previous triple repeat osmium-tannic acid fixation usually provided sufficient contrast within these ultrathin EM sections of chromosomes. Further staining with uranyl acetate and lead citrate was usually unnecessary. The sections were then examined with an Hitachi 7000 EM operated at 75 KV.

RESULTS

Using this "repeat chill" method yielded 100% separation of the flat-embedded chromosomes in the hardened plastic tissue blocks from the glass slide. This was successful in 8 consecutive trials. Metaphase chromosome spreads with pre-identified X chromosomes, including the fragile X chromosome [exhibiting fra(X)(q27.3)] were examined and photographed.

Figure 1 shows a partial metaphase from a longitudinal section through the entire spread so that all chromosomes from the same cell could be examined. The arrow shows the preidentified X chromosome from a full-mutation male individual's long-term, EBV-transformed lymphoblastoid cultures. The biotinylated α-satellite probe specific for the X chromosome centromere was hybridized to metaphase chromosomes and then detected with FITC-conjugated avidin. The chromosomes in Figure 2a exhibited orange fluorescence after propidium iodide staining while the X chromosome centromere was yellow. The fragile site appeared as a gap (arrow) in this preparation. This metaphase was sectioned and studied with TEM as shown at increasing magnifications in Figure 2b–d. Note the electron-dense centromeric regions in Figures 1 and 2b–d, indicated by arrowheads. An increase in density was also evident between sister chromatids as shown by the smaller arrows at the telomeric region of the fragile X chromosome short arm in Figure 2b,c. The fragile site (arrow) in Figure 2b–d also exhibited increased electron density and appeared as a narrow bridge or constriction between proximal and distal regions in the telomeric area of the long arm.

DISCUSSION

Previous TEM studies of an entire chromosome were confined to using whole mount preparations [Squarzoni et al., 1994]. Separating specimens in the hardened plastic embedding medium/epoxy tissue blocks from the surface of the glass slide or plasticware substrate was a previously insurmountable technical problem [Elliott et al., 1995]. In addition, the whole mount of the X chromosome was approximately 1.8 μm thick, which was not thin enough to obtain sufficient resolution for

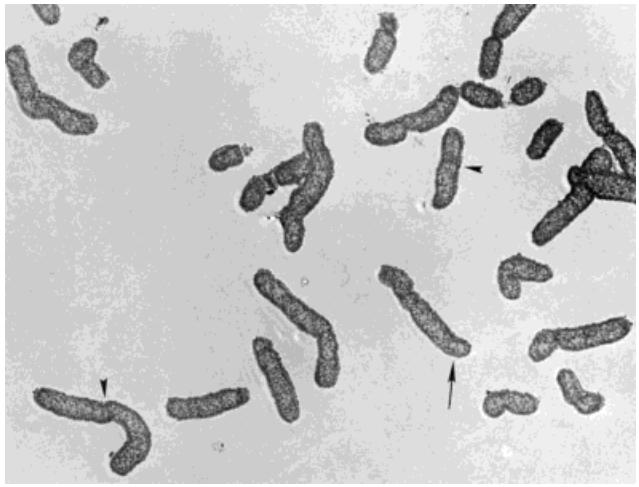

Fig. 1. Low magnification TEM micrograph of a partial metaphase spread. All chromosomes have been ultrathin-sectioned through their entire length. The arrow indicates the X chromosome preidentified by FISH. Arrowheads show examples of increased electron density at the centromere regions. (3,173×).

Fig. 2. **a:** FISH analysis of a metaphase spread where chromosomes were stained with propidium iodide (orange) and the fragile X chromosome centromere was hybridized with a biotinylated α-satellite probe and detected with FITC-conjugated avidin (yellow spot). The arrow points to the fragile site (which may appear as either a gap or break) on the X chromosome (1,414×). **b:** Transmission electron micrograph of the entire length of the same fragile X chromosome shown in **a** showing the electron-dense constriction at the fragile site (arrow). The electron-dense centromere (arrowhead) partitioned both short and long arms of the chromosome; note increased density between sister chromatids at the end of the short arm, also shown in **c** (smaller arrows) (9,310×). **c,d:** Higher magnifications of the same fragile X chromosome in **b** showing the electron-dense constriction at the fragile site appearing as a narrow bridge (arrow) connecting the distal and proximal parts of the long arm of the X chromosome. The electron-dense centromeric partition is indicated by arrowheads (16,734× and 25,500×, respectively).

internal structural analysis. TEM studies using ultrathin sections rather than whole mounts were confined previously to random focal regions of a chromosome [Rattner, 1987; Wandall, 1994].

We tried to overcome the technical problem of chromosome separation from the glass slide for the purpose of ultrathin chromosome sectioning. An attempt to heat the slide on a hotplate and to chill it with dry ice (CO_2) to separate the flat-embedded chromosomes in the hardened plastic tissue blocks from the glass slide surface was unsuccessful. With heating alone, the hardened plastic block easily separated from the glass slide and all the chromosomes remained on the slide. The dry ice method appeared insufficiently cold enough to allow separation of the chromosomes from the glass slide.

Liquid nitrogen was then tried instead of dry ice. Both the microscope glass slides and the tissue blocks frequently cracked into many pieces. However, we found that repeated immersion for 6–7″ in the liquid nitrogen to chill the slide/block interface allowed complete separation of the chromosomes from the glass. The differences in coefficients of contraction and expansion between glass and plastic allowed separation to occur after repeated chilling and warming.

When the glass slide was immersed in liquid nitrogen for 6–7″, approximately 25–50% of the flat-embedded chromosomes in the hardened plastic tissue blocks could be separated from the glass slide, which usually cracked. Following warming by either finger-touching or placing the slides for 2–3′ in a 70°C oven, the remaining tissue blocks were chilled again for 6–7″. This process was repeated until all of the broken pieces of glass were removed from the plastic tissue blocks.

FISH preparations were used for X chromosome preidentification, before TEM preparation. It is thought that G-banded preparations may also be employed with appropriate bright-field photomicrographic documentation.

This "repeat chill" method provided 100% separation and makes feasible the TEM study of chromosomes along their entire length by means of ultrathin sections. During the review of this paper, a question was raised as to how many of the 46 chromosomes per cell were analyzable by TEM. The theoretical answer is 100%, if there are no artifacts (including overlaps) in the chromosome preparation, and if none of the chromosomes are obscured by the "window bars" of the grid. The latter possibility can be overcome by using a "slot grid." It should be stressed that the only difference between our protocol and those that have been previously used for cytogenetic and EM studies is the use of liquid nitrogen to effect separation of the multiply fixed, plastic-embedded chromosomes from the glass slide. Since there was no evidence of shearing or tearing any of the chromosomes, it is thought that they were unaffected by the use of liquid nitrogen. Also, Elliott and colleagues [1995] reported no distortions of cellular ultrastructure after exposing multiply fixed, plastic-embedded cells to liquid nitrogen. Therefore, with this approach, the chromosome ultrastructural anatomy of centromeres, telomeres, fragile sites, and other areas can now be analyzed in relation to the structure and function of the chromosome and its components.

For example, the fragile site on the X chromosome obtained from an individual with a full FMR1 mutation and shown as a part of this technical TEM study, appeared as a narrow bridge or constriction connecting the proximal and distal parts of the long arm at Xq27.3, rather than several fibers as reported by Harrison et al. [1983], or as a gap or break in the bright-field light microscope. These fibers in the fragile site may represent dried protein fibers/residues on the surface of the fragile site and may also be equivalent to the chromatin fibers protruding from any surface (not limited to the fragile site) of the chromosomes as reported by Kattstrom and Nilsson [1992]. The bridge seen in TEM is narrow and deep and may not be visible with SEM. This may be related to the limitation of SEM providing resolution to examine the external structure of a chromosome but unable to uncover internal structures that require ultrathin sectioning of chromosomes. The appearance of the fragile site may vary from preparation to preparation. Future studies will be needed to determine whether the electron dense bridge is always present.

In addition, the centromeres in the ultrathin longitudinal sections of the chromosomes consistently exhibited increased electron density. This increased density was not apparent in previously reported studies of whole mount chromosomes [Messier et al., 1986, 1989; Squarzoni et al., 1994]. Whether the density of the centromere varies with the degree of chromosome condensation is as yet undetermined. Finally, additional information on the dynamics of kinetochore (centromere) and microtubule interactions [Wandall, 1994] within the context of the whole chromosome is now feasible with this repeat chill method.

ACKNOWLEDGMENTS

We thank librarian Lawrence Black for his assistance. We also thank Drs. James Ray and Maureen Sanz of North Shore University Hospital–Cornell University Medical College, for their constructive criticism during the preparation of the manuscript. In addition we thank Drs. Michael Partington and Gillian Turner for helpful discussions and enthusiasm during the planning of this project. This work was supported in part by the New York State Office of Mental Retardation and Developmental Disabilities and in part by grant MCJ360587 from the Maternal and Child Health Program (Title V, Social Security Act), Health Resources and Services Administration, Department of Health and Human Services.

REFERENCES

Elliott RL, Ledford LB, Head JF, Wang F (1995): A new method of preparing cultured cells for EM. USA Microsc Anal 13:15–16.

Harrison CJ, Jack EM, Allen TD, Harris R (1983): The fragile X: A scanning electronmicroscope study. J Med Genet 20:280–285.

Jenkins EC, Duncan CJ, Gu H, Genovese M, Krawczun MS (1992): Dialyzed fetal bovine serum increases cytogenetic fragile X expression. Am J Med Genet 43:155–160.

Kattstrom PO, Nilsson BO (1992): Preparation of human chromosomes for high resolution scanning electron microscopy. Arch Histol Cytol 55(Suppl):53–56.

Krawczun MS, Lele KP, Jenkins EC, Brown WT (1986): Fragile X expression increased by low cell-culture density. Am J Med Genet 23:467–473.

Krawczun MS, Jenkins EC, Lele KP, Sersen EA, Wisniewski HM (1990): Study of spindle microtubule reassembly in cells from

Alzheimer and Down syndrome patients following exposure to colcemid. Alzheim Dis Assoc Disord 4:203–216.

Messier PE, Jean P, Richer CL (1986): Easy transfer of selected mitoses from light to electron microscopy. Cytogenet Cell Genet 43:207–210.

Messier PE, Drouin R, Richer CL (1989): Electron microscopy of gold-labeled human and equine chromosomes. J Histochem Cytochem 7:1443–1447.

Rattner JB (1987): The organization of the mammalian kinetochore: A scanning electron microscope study. Chromosoma 95:175–181.

Sanchez-Sweatman OH, de Harven EP, Dube ID (1993): Human chromosomes: Evaluation of processing techniques for scanning electron microscopy. Scan Microsc 7:97–106.

Spurr AR (1969): A low viscosity epoxy resin and embedding medium for electron microscopy. J Ultrastr Res 26:31–43.

Squarzoni S, Cinti C, Santi S, Viamori A, Maradi NM (1994): Preparation of chromosome spreads for electron (TEM, SEM, STEM), light and confocal microscopy. Chromosoma 103:381–392.

Wandall A (1994): A stable dicentric chromosome: Both centromeres develop kinetochores and attached to the spindle in monocentric and dicentric configuration. Chromosoma 103:56–62.

Yao X-L, Jenkins EC, Wisniewski HM (1995): Study of mosaicism for chromosome 21 in skin fibroblast cultures from Alzheimer's disease and control individuals. In Iqbal K et al. (eds): "Research Advances in Alzheimer's Disease and Related Disorders." New York: John Wiley & Sons Ltd, pp 79–83.